Studies on Parasitic Protozoa.

II. (a) The Encystment of Rhizomastix gracilis Alexeieff; (b) Tetratrichomastix parisii n. sub-gen., n. sp.

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With Plates 31 and 32.

Introduction.

The intestine of that common grub, the larval Tipula, contains a rich bacterial flora, at the expense of which there flourish a surprising number of protozoa. Among these are at least eight flagellates and two amœbæ.[1]

It is customary to speak of such an intestinal fauna as parasitic, a word which implies that these organisms are in some sort harmful to their host. A short study of the actively moving and actively feeding protozoa in the gut of the crane-fly larva suggests much rather that the work they do in scavenging, and in keeping down the vast quantities of bacteria that threaten to choke the passage-way of the intestine, quite outweighs any trifling inconvenience that their presence might possibly cause the host. The largest and healthiest-

[1] Léger (1892) recorded three gregarines. I have already recorded, in a preliminary note ('Parasitology,' 1912), the following flagellates from Tipula: Trichomastix sp., Monocercomonas melolonthæ (Grassi), Polymastix melolonthæ (Grassi), Hexamitus intestinalis Duj., Embadomonas agilis Mackinnon, and E. alexeieffi Mackinnon. To these I would now add Rhizomastix gracilis Alexeieff and Tetratrichomastix parisii, n. sub-gen., n. sp. A paper dealing with the amœbæ is at present in preparation.

looking grubs were generally found to have the richest intestinal fauna. Probably such an association should rather be looked on as one of symbiosis. Certain of these protozoa have no doubt become closely adapted to their peculiar environment, and could flourish there only; but others of them, such as the flagellates Polymastix and Monocercomonas, are found in other larval insect hosts besides Tipula; while some are even less particular in their choice of host. The occurrence of these last is always sporadic and quite unpredictable. These are the "parasites facultatifs," which are of special value in helping us to fill in the gap between strict parasites and free-living forms.

To such a group of "facultative" parasites belongs the beautiful little monoflagellate, named by Alexeieff (1912) Rhizomastix gracilis. Alexeieff based his description on a single infection of an axolotl. I have recently found in Tipula a cercomonad which seems to me morphologically indistinguishable from Rhizomastix gracilis, and I see no good reason for "creating" a new species, even though the host be in the one case a vertebrate, and in the other an invertebrate. The infected leather-jackets were found in swampy ground near a stream—in much the sort of environment, that is to say, which would also be suitable for amphibians. I share with a few other protistologists the belief that animals totally unrelated, but frequenting the same feeding grounds, are liable to infection by the same "facultative" protozoan parasites. It would be well if the enthusiastic creators of "new species" would keep this possibility in mind.

The material at my disposal for the study of Rhizomastix was not much richer than that from Alexeieff's axolotls. Only two larvæ out of hundreds were found infected, and of these one so sparingly that only a dozen flagellates were found in the stained preparation. But in the other the organism was relatively abundant, and I found a large number of its cysts. I am thus able to add something to the little-known life-cycle of this cercomonad, and though the account is necessarily incomplete, I do not hesitate to publish it, believing as I do in the extreme interest to protistologists of such erratic parasitic forms.

(a) Rhizomastix gracilis Alexeieff.

The Flagellate Form.

A full description of the adult flagellate has been given by Alexeieff (1911). I quote the essential points. The form of the body is generally elongated, but may become globular; the posterior end is especially "metabolic." The single flagellum is about three times as long as the body; a small basal granule marks its emergence[1]; it is continued into the body by a sort of dark-staining filament, the rhizostyle, "qui atteint presque la longeur du corps et qui diminue brusquement de calibre en passant au voisinage du noyau, comme si une partie de la substance de la baquette s'arrêtait à la membrane nucléaire." The nucleus is very large, with voluminous karyosome, usually central; the peripheral chromatin is in the form of granules, connected with the karyosome by linin strands. The cytoplasm shows the clear alveolar structure characteristic of Cercomonas.

Alexeieff gives no measurements. The measurements I took showed the dimensions of the elongate flagellate individuals to be from $6\,\mu \times 3\,\mu$ to $11\,\mu \times 5\,\mu$. The diameter of the large round forms was about $6\,\mu$ to $8\,\mu$.

Figs. 1–7 (Pl. 31) show the appearance of the flagellate Rhizomastix gracilis as I found it in Tipula.

Encystment.

The oval bodies, "en bouteille, à goulot très court," which Alexeieff first described as the cysts of Rhizomastix (1911a), he referred later (1911b) to Chilomastix caulleryi.

[1] This basal granule mentioned by Alexeieff appears rather as a thickening of the rhizostyle than as a "granule"; sometimes it is possible to see a minute granule at the inner termination of the rhizostyle within the body of the organism, see figs. 2, 4 and 6.

The course of encystment seems to be as follows. The organism loses its flagellum (Pl. 31, fig. 8), but the rhizostyle usually persists. The body becomes rounded off, and there appear in the cytoplasm, hitherto finely alveolar, two or three large vacuoles (Pl. 31, figs. 9 and 10). These vacuoles run together, and form one very large vacuole, which lies to one side of the now spherical cyst. By this time a definite cyst-wall, "à double contour," has formed. The diameter of the finished cyst is 5 to 6 μ. The nucleus lies between the cyst-wall and the vacuole (Pl. 31, fig. 11). At this stage it still retains the characteristic features of the adult flagellate nucleus, with a large central karyosome, and peripherally arranged chromatin granules, from which linin threads extend towards the centre, like the spokes of a wheel. The rhizostyle is still clearly visible as a dark-staining line, often equatorial in position. About this time it would appear that chromatin escapes into the cytoplasm from the nucleus ; the cyst contents stain darkly, especially just within the cyst-wall. This suggests that possibly the nuclear material plays some part in the formation of this envelope.[1]

The nucleus seems to swell, and its outlines become vague. The karyosome breaks up into a number of small round masses (Pl. 31, figs. 12 and 13). A spindle now makes its appearance, on which the karyosome granules occupy the position of the equatorial plate (Pl. 31, figs. 14 and 15). It is not clear what becomes of the peripheral chromatin. Possibly it unites with the central mass, but as the cytoplasm stains even more intensely and contains more dark granules than before, I think it possible that a good deal of the peripheral chromatin is absorbed.

Though the spindle is perfectly well defined, I have found no indication of centrioles at its poles. In one cyst (Pl. 31,

[1] Cf. Alexeieff's observations (1913A) on the part played by chromidia in the cyst-formation of Chilomastix and other protozoa. Hartmann and Whitmore (1910) think that the extrusion of chromatin from the nucleus of Entamœba coli has something to do with the formation of the large central vacuole in the cyst.

fig. 16) there certainly appeared to be a group of granules at each end of the spindle, but this was probably chromatin that had travelled there from the equatorial plate, or else the residue of the peripheral chromatin.[1]

Gradually the spindle lengthens, and the chromatin from the middle separates towards the two poles, there forming dense, dark-staining, club-shaped masses. The spindle extends right across the cyst, curved against the bulge of the vacuole. It thins out and becomes vague, then disappears, leaving the two nuclei. These are at first exceedingly small and compact (Pl. 31, fig. 20), but they presently swell out, and take on the characters of the normal flagellate nucleus, with peripheral chromatin and a voluminous karyosome. As a rule the karyosome is not central, but lies pressed against the nuclear membrane(Pls. 31 and 32, figs. 22–25). The daughter-nuclei sometimes lie close together, sometimes at opposite sides of the cyst, which now is inclined to lose its regular circular outline (Pl. 32, fig. 26).

The rhizostyle has meanwhile divided into two. Sometimes this division takes place very early, even before that of the nucleus, sometimes later. This division does not seem to be a splitting of the whole structure; division of the basal granule is followed by the gradual growth of a second rhizostyle from one of the daughter-granules (Pl. 31, figs. 13, 23 and 24).

[1] If the recent "systematisation" of primitive mitosis given by Alexeieff (1913) is to be followed, then the mode of nuclear division in the cysts of Rhizomastix gracilis comes near to that named mesomitosis. "Centrioles aux deux pôles du noyau en division; la plaque équatoriale, formée par le caryosome, massive au début, se morcelle en un certain nombre de chromosomes qui . . . se repartissent aux deux pôles où ils se confondent avec les centrioles." But I have been unable to demonstrate centrioles in Rhizomastix, and in this respect Alexeieff's rheomitosis, in which the centrioles are early hidden by accumulating chromatin granules, seems to meet the case better—"à la place de centrioles on observe des corps chromatiques de plus en plus volumineux et finalement on se trouve en presence d'une apparence de promitose avec ses corps polaires."

This stage, with two large nuclei, a large vacuole, and clear alveolar cytoplasm, is much the most abundant. I have looked in vain in my small material for the fission of the cytoplasm which I believe follows, and for the escape of the two flagellates. Probably the cysts are passed out from the gut of the host in this binucleate state.

In the second of my preparations, where there were no cysts seen, ten of the twelve small flagellates were found with the karyosome eccentric, and possibly they had recently emerged from cysts (Pl. 31, figs. 4–7; Pl. 32, figs. 27–29). In these forms it was often impossible to trace the rhizostyle past the nucleus, into the anterior border of which it appeared to be inserted. (Cf. the flagellar apparatus in Oicomonas [see Hartmann and Chagas, 1910], to which this arrangement is very similar.) In one small individual (Pl. 32, fig. 29) there were three nuclei, but I do not know what this indicates.

In the cysts I have found no trace of fusion of the nuclei, nor any indication that there is a sexual process there. They seem to be simply multiplication cysts.

Systematic Position.

As Alexeieff suggests (1911A), the affinities of Rhizomastix are probably on one side with free-living forms like Oicomonas and Cercomonas,[1] and on the other with parasites like Herpetomonas and its allies. There is a striking resemblance between Oicomonas, as figured by Hartmann and Chagas, and certain flagellate stages of

[1] I am unable to appreciate Alexeieff's reasons (1911B) for removing Oicomonas from among the Cercomonadines. He speaks of its mode of encystment as resembling that of Monas. At present our knowledge of the encystment of Oicomonas and its allies is so scanty as to afford little reliable indication of their affinities, but I do agree that in the comparative study of such processes lies our surest hope of ultimately bringing something like order out of the present chaos of flagellate classification. "Die Kenntniss der Entwicklung ist das erste Postulat der Protozoenforschung" (Schaudinn).

Rhizomastix. The rhizostyle of Rhizomastix is very possibly homologous with the much-debated fibril "Doppelfaden," that Prowazek was the first to demonstrate in Herpetomonas muscæ-domesticæ.

My account of the encystment does not add much to help in determining the systematic position further. Cysts of Cercomonas have been described by Hartmann and Chagas (1910), Wenyon (1910), and Alexeieff (1911), but those seem in every case to be mere "Schützcysten," in which no division phenomena take place. Doflein (1910) and Ogawa (1913) have described and figured certain globular forms which occurred in their cultures of Trypanosoma rotatorium. I have been struck by the resemblance of these to the cysts of Rhizomastix. One of Doflein's figures in particular shows a large vacuole filling out the bulk of the sphere, and crushing aside the cytoplasm, which contains two nuclei. There are kinetonuclei present, of course, and there is no cyst-wall; in other respects the picture is not unlike what I have shown in Pl. 32, fig. 26. Ogawa describes these as "Ruhe oder Dauerformen."

It is very difficult to say how far a similar mode of nuclear division indicates affinity between protozoa. The division of Rhizomastix within the cyst does not bear much resemblance to that of Cercomonas flagellates as described by Hartmann and Chagas and others. On the other hand, the division of Trypanosoma lewisi is a mesomitosis according to certain authors.

If importance is to be attached to such points as these, then the study of Rhizomastix gracilis tends to strengthen the view that the Trypanosomidæ Doflein (= Herpetomonadidæ Alexeieff) are derived from Cercomonadine ancestors.

(b) Tetratrichomastix Parisii, n.sub-gen., n. sp.

General Considerations.

Though the trichomonad flagellates have received much attention from a number of workers during the last few years, there is still considerable difference of opinion as to the systematic value of the flagellar apparatus. On the one hand, Doflein (1911) considers that the genera Trichomonas and Trichomastix are just varieties of the same form, of which the recurrent flagellum is attached to the body-wall in the first case, while it remains free in the second. On the other hand, there are those, like Parisi (1910), who think that the number and mode of attachment of the flagella are points of real value to the classifier of these organisms. Parisi, accordingly, has split up the genus Trichomonas Donné into three sub-genera :

(1) Trichomonas sensu stricto, with three anterior flagella, and an undulating membrane.

(2) Tetratrichomonas, with four anterior flagella, and an undulating membrane.

(3) Trichomastix, with three anterior flagella, and one recurrent flagellum, which does not adhere to the body to form an undulating membrane.

The flagellate which I am about to describe from Tipula, while undoubtedly a trichomonad, falls into none of these three sub-genera. It is provided with five flagella, of which four are directed forwards, and the fifth is a "Schleppgeissel," as in Trichomastix. This form, then, bears exactly the same relations to Trichomastix that Tetratrichomonas has to Trichomonas.[1] I propose to place it in a fourth sub-genus, Tetratrichomastix. This seems the simplest method of proceeding, so long as we abide by the present classification of the trichomonads.

To subdivide the genus is certainly a convenience, and the characters on which the subdivision is based seem clear enough. What is less satisfactory are the "species" within these sub-genera. I have already expressed my scepticism with regard to what may be called "host-species" in parasitic protozoa. Some authors consider that flagellates should

[1] A true Trichomastix with three anteriorly directed flagella, indistinguishable from Trichomastix trichopterorum, Mackinnon, also occurs in Tipula (see Mackinnon, 1912).

be classified upon morphological grounds alone. Alexeieff (1911) gives five characters, which, taken together, should form, he considers, a reliable basis for classifying the species of Trichomonas. These deal with such points as the dimensions of the axostyle, the structure of the nucleus, the distribution of the extra-nuclear siderophilous granules, etc., and they must be studied, he says, on the adult flagellate. Now these are characters that are subject to some fluctuation, particularly if the organism be a parasite of more than one kind of host; moreover, it is by no means always easy to decide which is the typical adult form. In the case of Trichomastix, two of the said characters, i.e. the parabasal body and the condition of the undulating membrane, are necessarily absent, and this renders the species determination on morphological grounds increasingly uncertain. What makes the difficulty still greater is that slightly stronger differentiation of iron-hæmatoxylin preparations produces such difference in the degree to which the "species" characters are exhibited.

In the present case two forms of Tetratrichomastix occur (vide infra), but I hesitate to dub them species. As these two forms of the flagellate never occur side by side in the same preparation, I am strongly of the opinion that the degree of intensity of the staining must be taken into account. For the present I propose to consider them as forms of one flagellate—Tetratrichomastix parisii, n. sub-gen., n. sp.

Diagnosis.

Tetratrichomastix n. sub-gen. Flagellate with axostyle and five free flagella, four anteriorly, and one posteriorly directed.

T. parisii n. sp. Axostyle slender and rather poorly developed. Nucleus oval or round, (1) compact, rich in chromatin blocks (form A, Pl. 32, figs. 30 and 31), or (2) feebly siderophilous, with a few scattered chromatin granules and a well-defined nuclear membrane (form B, Pl. 32, figs. 32 and

33). In form A the nucleus is often surrounded by a halo of small, siderophilous, drop-shaped bodies (ingested bacteria?). In form B, the cytoplasm contains several large vacuoles, and stains relatively intensely. No cytostome visible. Dimensions 8 μ by 4 μ to 12 μ by 7 μ.

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EXPLANATION OF PLATES 31 AND 32,

Illustrating Miss D. L. Mackinnon's paper on (a) "The Encystment of Rhizomastix gracilis Alexeieff; (b) Tetratrichomastix parisii n.sub-gen., n.sp."

[Figures drawn to scale (× 4000 ca.) under Zeiss comp. oc. 12 and 2 mm. apochromat. Figs. 7, 8, 27, 28 and 29 are on a slightly smaller scale than the rest. The stain employed was Heidenhain's iron-hæmatoxylin, after fixation with sublimate alcohol.]

Figs 1–29. Rhizomastix gracilis.

Fig. 1.—Large pear-shaped flagellate individual, showing single long flagellum (*Fl.*), "rhizostyle" (*Rh.*), basal granule of Alexeieff (*B. G.*), and large vesicular nucleus (*Nu.*).

Fig. 2.—Flagellate of more spherical form. The granule at the inner end of the rhizostyle is well shown here (*G.*).

Fig. 3.—Flagellate of which the nucleus has an eccentric karyosome.

Figs. 4–7.—Various forms assumed by Rhizomastix gracilis. Note the clear zone round the nucleus, probably an artefact and due to shrinkage. In figs. 4, 5 and 6 the cytoplasm shows numerous inclusions, probably bacteria. In figs. 5 and 7 the rhizostyle has much the same relations to the nucleus as in Oicomonas.

Fig. 8.—Rounded-off individual without flagellum. Resting stage?

Fig. 9.—Preparation for encystment. The flagellate rounds off, and its cytoplasm becomes more coarsely vacuolated.

Fig. 10.—A rather later stage. The flagellum has disappeared; the vacuoles become larger and fewer in number.

Fig. 11.—The cyst formed. Note the large vacuole (*Va.*) and the persistent rhizostyle (*Rh.*).

Fig. 12.—The nucleus swells, and there is an escape of chromatin into the cytoplasm.

Fig. 13.—The nuclear outlines become vague; the karyosome fragments; the rhizostyle has doubled.

Figs. 14–16.—Formation of the spindle. In fig. 16 a certain amount of chromatin has already collected at the poles.

Figs. 17–20.—The chromatin collects at the poles. The spindle thins out more and more.

Fig. 21.—The spindle has disappeared. Two compact nuclei are left.

Figs. 22–26.—Various aspects of the cyst with two nuclei. In fig. 22 the large vacuole has disappeared. In fig. 26 one of the nuclei has taken on the vesicular form, and the cyst has elongated.

Figs. 27–29.—Small flagellates with eccentric nucleus. Possibly these have recently emerged from cysts. Fig. 29 shows an individual with three nuclei.

Figs. 30–33. Tetratrichomastix parisii.

Fig. 30.—Form A. Note fully developed axostyle and dark-staining nucleus with large chromatin masses.

Fig. 31.—Form A. Note halo (*H.*) of drop-shaped bodies surrounding nucleus.

Fig. 32.—Form B. Note relatively large, faintly staining nucleus, poor in chromatin.

Fig. 33.—Form B. Flagellate without axostyle.